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A Second Generation Biofuel from Cellulosic Agricultural By-product Fermentation Using *Clostridium* Species for Electricity Generation

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Abstract

The production of second generation biofuel is essential for limiting food versus fuel competition. Butanol is one of the important biofuel for the future. Agricultural by-products namely bagasse and potato peel were hydrolyzed to produce readily fermented sugar for butanol fermentation. The butanol concentration was 1 – 2 g/l. To test the electricity generation, a customized generator was used for butanol combustion. The electricity produced was up to 1300 watts. Further improvements are needed in the hydrolysis method, medium composition, and generator design. This research has demonstrated that bagasse and potato peel are potential feedstock for producing butanol for generating electricity

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Keywords: butanol; *Clostridium acetobutylicum*; second generation biofuel; agricultural by-products; electricity

1. Introduction

The world petroleum consumption in 2012 was estimated at 89 million barrels per day [1], nearly half of it was for producing gasoline. Our energy demand is projected to increase by more than 50 % by 2025 [2]. However, the

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current oil reserves are estimated to run out within the next 50 years. Therefore, the production of fuels from renewable resources such as biofuel is vital to secure our energy needs. One of the most widely used biofuel is ethanol from carbohydrates [3]. Global ethanol production is reaching 1.5 million barrel per day [1]. However, ethanol has several drawbacks [4, 5]. Its energy content is only 70 % the energy of gasoline. Also, the construction of new pipelines and storage infrastructure are needed because ethanol is hygroscopic and corrosive.

The current problem in ethanol production is the use of food sources such as corn or sugar cane as the feedstock. This 1st generation biofuel production is not sustainable due to the limited food supply and the increasing of world population. The 2nd generation biofuel have been developed to limit the fuel versus food competition. The raw materials used are non-food biomass such as agricultural by-products (sugar cane bagasse, rice straw, and potato peel), wood chips, and grass.

Butanol (C_4H_{10}) is a biofuel that has several advantages over ethanol. It has similar properties to gasoline [4]. Also, butanol less corrosive than ethanol [6]. Therefore, it can easily mixes with gasoline, making it compatible with the existing pipelines and storage facilities. Furthermore, butanol heat of vaporization is only half of ethanol's. Consequently, the engine running on butanol is easier to start during cold weather than the one running on ethanol. Butanol was initially produced from carbohydrate fermentation by *Clostridium acetobutylicum* in the process known as acetone-butanol-ethanol (ABE) fermentation with a product ratio of 3:6:1[5]. During 1970s, the process was replaced by chemical catalytic process from propylene. However, the increasing demand of butanol as biofuel has revived the butanol fermentation process. Several chemical giants such as DuPont, BP, GEVO has planned to build butanol fermentation plant or transform ethanol plant to butanol [7]. Several attempts have been performed to improve butanol productivity via metabolic engineering and improvement of process technologies [4]. But, there are only some researches on using cellulosic feedstock for butanol fermentation.

Our research used the biomass from agricultural waste for butanol fermentation using *Clostridium acetobuylicum*. Bagasse and potato peel were selected since these are widely availability in Indonesia. Bagasse hydrolysate was prepared using alkali and enzymatic treatment. Potato peel hydrolysate was prepared using fermentation by the fungi *Aspergillus niger*. Butanol was used to generate electricity.

2. Material and methods

2.1. Bacterial strain and stock preparation

Clostridium acetobutylicum ATCC 824 was obtained from Center for Biotechnology Studies, University of Gadjah Mada, Yogyakarta. The stock was prepared by growing the freeze-dried bacteria in the Reinforced Clostridial Medium (RCM) (Oxoid, Hampshire, UK) inside a 2.5 liter anaerobic jar (Oxoid, Hampshire, UK). The stock is maintained in the medium and stored at 4°C in 25-ml McCartney bottles. *Aspegillus niger* AspF2 was obtained from Department of Biology, Universitas Pelita Harapan. The fungus was stored on PDA agar.

2.2. Biomass hydrolysis

The hydrolysis of bagasse was performed using the following steps. Dry bagasse was obtained from Kebon Agung Sugar Mill in Malang, East Java. Ten grams of bagasse was suspended in 500 ml of 1 % NaOH. The mixture was then autoclaved at 120°C for 30 min at 2 bars. The liquid was removed using filter paper to separate hydrolyzed hemicelluloses and lignin. The retained solid was washed with water. The cellulose in the solid was enzymatically hydrolyzed using cellulose from *Trichoderma viride* (Sigma Aldrich, USA) [8]. The pre-treated biomass solid (about 20 g/l) was suspended in 12.5 mM acetate buffer at pH 5 for 8 hours. The amount of enzyme used was at 500 U.

The potato peel hydrolysate was prepared by autoclaving 20 g of the peel followed by milling to produce the slurry. This potato peel slurry was inoculated with A. niger and incubated for 7 days at 30°C. The fermented slurry was later diluted with 100 ml of water. The final product was obtained by pressing the suspension through a filter.

2.3. Medium and cultivation

The bacteria were cultivated in four separate media. Medium A was a rich RCM. Medium B was a mineral medium based on the previous report [9], containing in 1 liter medium: glucose, 20 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.38 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeSO_4.7H_2O$, 0.01 g; $MnSO_4.H2O$, 0.01 g; NaCl, 0.01 g; p-aminobenzoic acid, 0.001 g; and biotin, 0.00001 g. Medium C and D contained the same composition as medium B, except the glucose was substituted by the bagasse hydrolysate and potato peel hydrolysate, respectively.

Inoculum was prepared in a McCartney bottle containing 20 ml culture. The main fermentation was performed in a 500-ml gas washing bottle (Duran, Germany) containing 200 ml culture. A pair of 0.22 μm sterile membrane filter was placed at gas inlet and outlet. Anaerobic condition was maintained by sparging the culture with nitrogen during the sampling process. An anaerobic indicator paper containing resazurin was used to monitor the oxygen level. During sampling, a roughly 10 ml culture was taken for OD, pH, sugar, and alcohols analyses. Supernatant was obtained by centrifugation at 5000 g for 5 min.

2.4. Growth and metabolite analysis

The cell growth was monitored by measuring OD at 600 nm (Human spectrophotometer, Korea). Glucose and other reducing sugar were analyzed using DNS method. Ethanol and butanol were analyzed using Gas Chromatography (Shimadzu GC-14A using SUPELCOWAX-10 type column (Supelco Inc., 0.53 mm ID, 15 m, 0.5 mm) with temperature of 50°C and a Flame Ionization Detector.

2.5. Electricity generation

A modified generator was designed for electricity generation from butanol. The generator has concentric ferromagnetic steel. The contra flow heat exchange occurred at the outer shell to vaporize the liquid fuel by using the heat from exhaust gas. The combustion occurred at the inner chamber. The generator has a capacity up to 6000 watts with the fuel consumption of 1 liter/hour.

3. Results and discussion

3.1. Bagasse hydrolysis

The biomass concentration after the NaOH hydrolysis was reduced from 20 g/l (2 % w/v) to 8.9 ± 0.5 g/l. This removed 55 % of the solid mass, mainly as lignin and hemicelluloses since cellulose is more stable against strong basic at low concentration. Previous report shows that the optimum NaOH concentration of 1 % for 30 min removes 49 % of initial solid [10]. The fraction of lignin and hemicelluloses removed roughly 75 % and 25 %, respectively. The enzymatic hydrolysis of cellulose in bagasse resulted 13.5 ± 0.2 g/l reducing sugars/liter in a total of 200 ml solution. This sugar concentration is similar to the value previously reported [11].

The initial amount of biomass prior to hydrolysis by NaOH was 10 g, making the sugar overall recovery is 27 %. The recovery based on the NaOH pre-treated biomass is 61 %. Previous result of enzymatic hydrolysis using cellulase in coastal grass and bagasse recovered 71 % [10] and 50 % [11] sugar from cellulose, respectively. These values may explain that the enzymatic hydrolysis needs to be optimized further, such as by addition of cellubiase.

The reducing sugar concentration from potato peel hydrolysate was 4 g/l. Potato peel water content is estimated at 80 % [12]. This gives the sugar overall recovery of 10 %. *A niger* posseses an array of extracellular enzymes for long chain carbohydrates including cellulase, xylanase, amylase, and glucoamylase [13]. The low recovery may be due to the limitation in the enzymes activity in this solid state fermentation. While it is less expensive, the solid state fermentation technique has a drawback of a low substrate and metabolite transport.

3.2. Butanol production

Butanol produced during fermentation by *C. acetobutylicum* is presented in Table 1. The highest butanol concentration was obtained from the fermentation in medium A (RCM), a rich medium that is designed for optimum growth of *Clostridia*. Typical butanol concentration from rich media is between 7 to 12 g/l [14]. This is due to feedback inhibition by butanol when the total solvent (acetone, butanol, ethanol) produced has reached 20 g/l [15]. A butanol tolerant mutant strain was able to reach 18 g/l [16].

Butanol production in mineral medium and hydrolysates were much lower than in medium A, probably due to an absent of complex nitrogen sources such as yeast extract and peptone that are essential for optimum growth and solvent production [4] and also due to a low sugar concentration. Our result in medium A also shows the importance of complex nitrogen sources. Previous report shows that butanol concentrations from the fermentation with initial sugar of 10 – 20 g/l are 2 – 4 g/l since a low sugar concentration may permits the accumulation of butyrate which competes with butanol synthesis [9]. These relatively low sugar concentrations in medium B, C, and D are inevitable due to some restriction in the hydrolysis steps such as the problem in suspending the biomass at a high concentration before hydrolysis. The butanol yields obtained from the fermentation in medium C and D are close to the theoretical yield (41 g/100 g glucose). The lower yield in medium B shows that butanol fermentation is not completed.

Table 1. Butanol and ethanol production in different medium with the initial sugar concentration is given (the data was from 74-77 hours of fermentation).

Medium	Initial sugar (g/l)	OD_{600}	Butanol (g/l)	Butanol yield (g/100 g sugar)
A/ RCM	5.0	0.45	7.7	n.a[†]
B/ Mineral medium	20.2	0.41	1.8	31.0
C/ Bagasse hydrolysate	12.7	0.55	2.2	43.6
D/ Potato peel hydrolysate	3.3	n.a[‡]	0.9	37.1

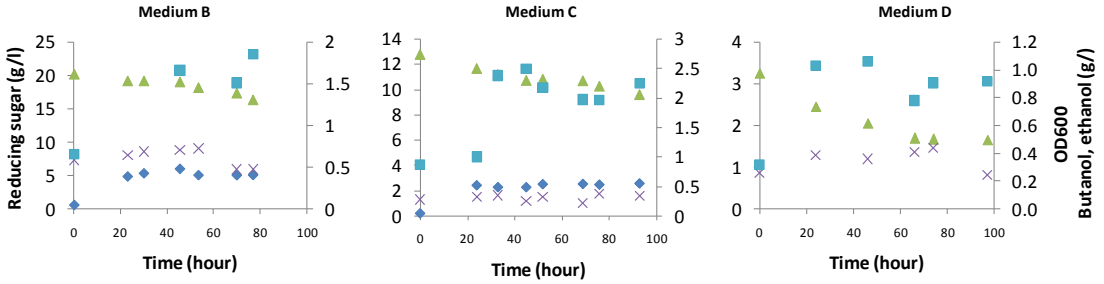

Fig 1. Cell growth (♦), butanol (■) and ethanol (×) production, and sugar consumption (▲) during fermentation of medium B, C and D. The growth measurement in medium D is not available due to the dark color from the browning reaction in the medium during autoclaving.

Figure 1 shows the time course of the fermentation in medium B, C, and D. The growth phase lasted for 1 day followed by butanol synthesis. The large part of sugar was not metabolized may be because of the relatively low cell density. The low cell density (OD_{600} <1) is probably due to a low chloride concentration at only 10 mg/l. The addition of chloride may improve cell growth; subsequently increase butanol productivity but not the maximum butanol concentration. The ethanol level in the medium B, C, and D were about 0.3-0.4 g/l.

[†] RCM contains other carbon sources such as beef extract, yeast extract, and peptone.
[‡] The OD measurement is not available due to the dark color of the medium

3.3. Electricity generation

The power generated from butanol fuel is shown in Table 2. Nearly 1300 watts of electricity was generated. The use of low purity butanol only slightly reduced the power. However, the energy efficiency and power are lower than the efficiency from gasoline and ethanol combustion. One reason is that butanol has a high boiling point at 118°C, compared to gasoline (50-85°C) and ethanol (78°C). The outer shell in the generator needs to be modified to provide a better heat exchange that allows more efficient butanol vaporization and reduces the loss of butanol in the exhaust gas.

Table 2. The performance of generator using 100 ml of butanol at different purity, compared with gasoline and ethanol.

Fuel	Power (W)	Energy produced (kJ)	Efficiency (%)
Gasoline, pure	4000	1,044	16.2
Ethanol, 96 %	1855	593	15.8
Butanol, 99 %	1260	288	10.0
Butanol, 90 %	1190	240	9.1
Butanol, 80 %	1120	215	9.2

4. Conclusion

Our research has successfully utilized the agricultural by-product (bagasse, potato peel) for fermentation to produce butanol. The generator has been designed for producing electricity from butanol combustion. Several improvement strategies still have to be pursued. These include the development of a high concentrated biomass hydrolysis to produce high sugar concentration, the fermentation technology improvement by modifying the medium composition, and the tuning up of the generator to increase energy efficiency. Regardless the modest result, the research has produced a platform for a future optimized hydrolysis, fermentation, and power generation strategy in a larger scale.

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